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Strategic Environmental Research and Development Program

Natural Attenuation of Explosives in Soil and Water Systems at Department of Defense Sites: Final Report

by *Judith C. Pennington, Paul H. Miyares, David B. Ringelberg, Mansour Zakikhani, C. Michael Reynolds, Debra Felt, Richard B. Coffin, Douglas Gunnison, Luis Cifuentes, Herbert L. Fredrickson, Thomas F. Jenkins*

Prepared for Strategic Environmental Research and Development Program
Arlington, VA

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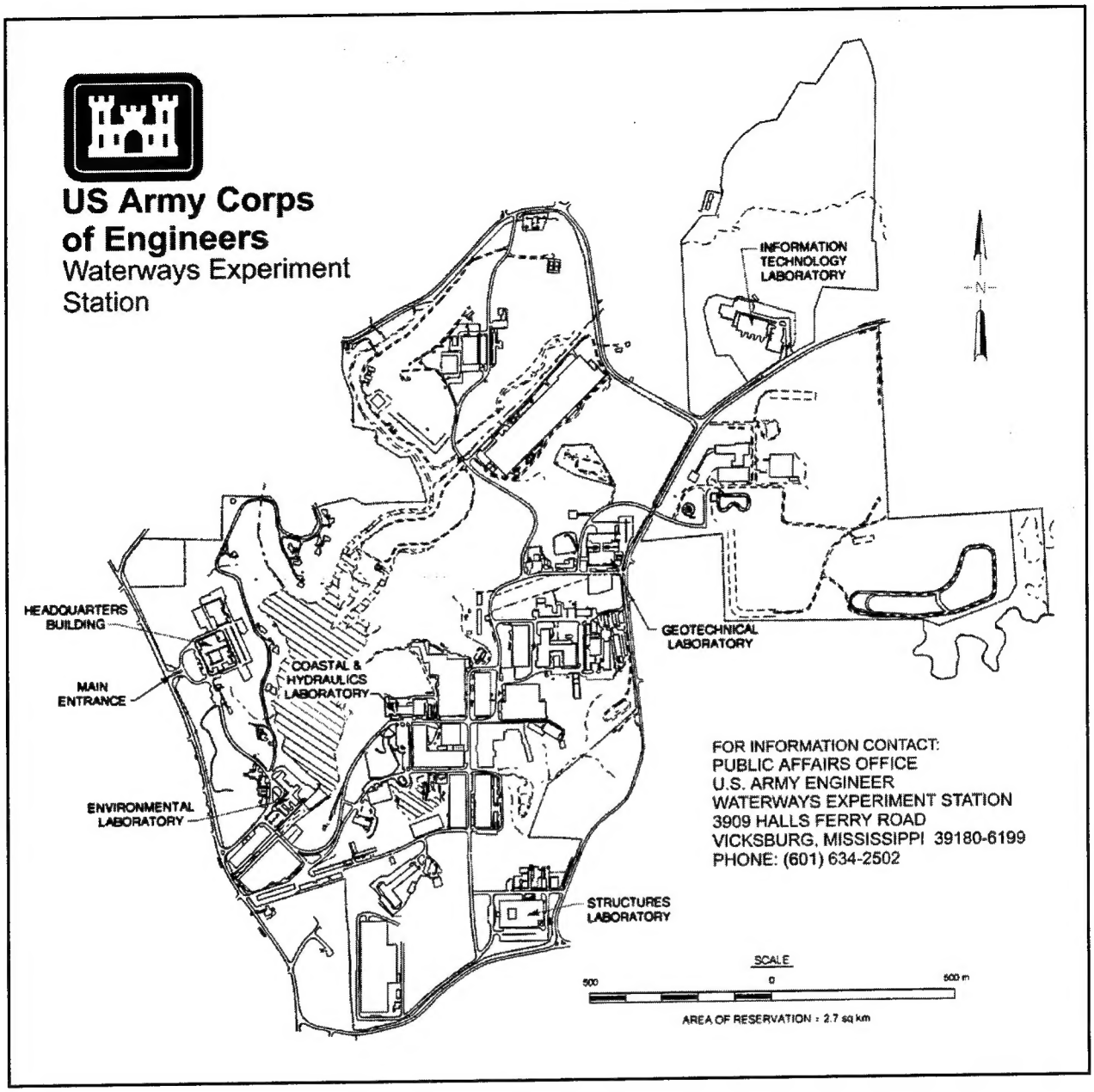

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Preface

This report was prepared by the Environmental Laboratory (EL), U.S. Army Engineer Waterways Experiment Station (WES), Vicksburg, MS, a complex of five laboratories of the U.S. Army Engineer Research and Development Center (ERDC), in association with the U.S. Army Cold Regions Research and Engineering Laboratory (CRREL), Hanover, NH, another of the ERDC laboratories; AScI Corporation, McLean, VA; Texas A&M University (TAMU), College Station, TX; and the Naval Research Laboratory (NRL), Washington, DC. The research was sponsored by the Strategic Environmental Research and Development Program, Arlington, VA, Dr. John Harrison, EL, and Mr. Bradley P. Smith, Arlington, VA, Executive Directors, Project Number CU1043. The Principal Investigator was Dr. Judith C. Pennington, EL.

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1 Introduction

Background

The regulatory community and the general public are becoming increasingly aware that solutions to environmental contamination are not as simple as imposing stringent regulations. Environmental remediation technology is evolving toward more practical goals incorporating less expensive, less intrusive, long-term solutions. Natural attenuation may be a legitimate and sensible alternative to existing remediation techniques if appropriate implementation guidance is developed. Research in support of development of guidance for selection and implementation of natural attenuation for the explosives 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) was sponsored by the Strategic Environmental Research and Development (SERDP), Environmental Security Technology Certification Program (ESTCP), and U.S. Army Industrial Operations Command. The research focused on the following areas: (a) groundwater monitoring, (b) site capacity estimation parameters, (c) biomarkers, (d) stable isotopes, and (e) modeling. Several reports are available describing results of those efforts (Pennington, Zakikhani, and Harrelson 1999; Pennington et al. 1998a, 1999a, b, c). The SERDP portion of the research focused specifically on the development of biomarkers, stable isotopes, and modeling of attenuation processes. An interim report describing results of the project through Fiscal Year 1998 (FY 98) included results in all of these research areas except stable isotopes (Pennington et al. 1999b). The approach and results for each area presented in the interim report are summarized in the following paragraphs.

Groundwater monitoring

Declining concentrations of explosives in groundwater over time may be evident from site historical data. If adequate, these historical records provide the first line of evidence under the U.S. Environmental Protection Agency (EPA) (1999) policy directive, "Historical groundwater and/or soil chemistry data that demonstrate ... declining contaminant mass and/or concentrations...." The first task was to evaluate the extensive historical data at Louisiana Army Ammunition Plant (LAAP), Doyline, LA, for trends in explosives concentration. Since long-term monitoring is required to verify any observed trends, development of a

monitoring plan was the next task. To optimize the validity of trends, attention was focused on the quality of the data generated by the groundwater monitoring plan. Special emphasis was placed on development of techniques for assuring quality data. Efforts included techniques for minimizing the influence of oxygen at the wellhead on sampling formation water; maintaining sample integrity; sample preservation; precision, accuracy, and representativeness of the data; data comparability; field quality control; and confirmation of analytical chemistry. For monitoring data to support the second line of evidence, "hydrogeologic or geochemical data that can be used to indirectly demonstrate the type(s) [and rate] of natural attenuation processes active at the site...," collection of data that was not collected during previous monitoring was required. Therefore, an extensive list of explosives transformation products and geochemical parameters was included in the analyses. The monitoring plan developed for the demonstration site, LAAP, was validated at a second site, Joliet Army Ammunition Plant (JAAP), Joliet, IL.

Declining concentrations of explosives over the 2-year monitoring period were documented at LAAP. Results support the first line of evidence required under EPA guidance for verification of monitored natural attenuation, i.e., declining contaminant mass. Methods were developed to optimize accuracy and minimize variability between sampling events, so that trends in concentration over time were readily demonstrated and reliable. None of the geochemical characteristics of the site correlated with explosives concentrations. Therefore, monitoring geochemical parameters provided no evidence of natural attenuation processes at LAAP. Sampling methods developed at LAAP were verified by application at JAAP. Although the sampling period at JAAP was limited to 9 months, about 20 percent of the wells exhibiting concentrations of explosives above detection limits showed significant declines. Geochemical parameters were unrelated to explosives concentrations as observed at LAAP.

Site capacity

Site capacity for attenuation of explosives is a function of soil sorption, biodegradation, transformation, and chemical interactions with soil organic matter and clay. Not all of these processes are well-defined for explosives. Nonetheless, site capacity can be measured by simple batch shake and column tests, which quantify contaminant half-life and adsorption coefficients. These capacity measurements can be used to refine predictive capabilities of numerical models for the site. Both batch shake and column tests were performed on soils from the LAAP aquifer to quantify sorption coefficients and disappearance rates for explosives. These data can support the second line of evidence by providing attenuation rate measurements.

Batch shake test results were demonstrated to adequately describe sorption and disappearance rate constants in the LAAP soils. Sorption of explosives compounds by the aquifer soils was limited, with all constituents showing partitioning coefficient K_D values below 1 L kg^{-1} for all soils. These results indicated that a single average sorption coefficient for each compound in the

LAAP soils adequately described sorption for numerical modeling. Disappearance rate constants were low in comparison with those typically observed in surface soils. Use of the disappearance rate coefficients in modeling was complicated by the proximity of the coefficients to zero and the uncertainty that this created about applying results from short-term bench scale testing to field scale. Use of the disappearance rate coefficients in groundwater models may require adjustment to accurately depict measured groundwater concentrations that reflect field conditions and a longer time frame than is possible with bench scale batch and column studies. These results suggest that mass transport limitations rather than site capacity restrict transport at LAAP.

Biomarkers

Biomarker techniques have been used to detect the involvement of microorganisms as biocatalysts for the transformation and/or degradation of contaminants. The techniques have been used extensively to measure changes in microbial community structure and microbial response to contaminants even after the contaminant is no longer detectable (e.g., the plume has migrated beyond the microbial community, or the contaminant concentration has been reduced to levels below detection). Radiorespirometry provides direct evidence that transformation and/or complete mineralization can occur in site soils by challenging the indigenous microflora with radiolabelled TNT and/or RDX. Mineralization is evidenced by evolution of radiolabelled carbon dioxide. Analyses of bacterial polar lipids provide information on microbial community biomass and composition and the changes resulting from anthropogenic chemical perturbations. Analysis of nucleic acids provides a mechanism to detect the presence of genes encoding enzymes required for explosive degradation in situ. The radioassay results can be correlated with results from the two biomarkers and site geochemical data to build a case for natural attenuation onsite. Biomarkers demonstrate whether (a) a viable biomass is present, active, and capable of metabolizing RDX and TNT, (b) catabolic genes necessary for in situ degradation are present, and (c) significant positive correlations to explosive concentrations, geochemistry, and mineralization properties exist. The third line of evidence "Data from field or microcosm studies ... which directly demonstrate the occurrence of a particular natural attenuation process ... to degrade the contaminants of concern..." generally requires laboratory testing of field samples (groundwater and/or aquifer material). Therefore, samples of aquifer soils collected by cone penetrometry were subjected to biomarker analyses in laboratory microcosms. Soils from LAAP were used for development and refinement of techniques while soils from JAAP were used to validate procedures. The two biomarker techniques, phospholipid fatty acids (PLFA) analysis and microbial DNA analysis, were coupled with radiorespirometry in microcosm studies.

Integration of results from radiorespirometry, nucleic acid, and lipid biomarker techniques was used to evaluate the ability of indigenous microorganisms to degrade explosives. For the two sites, LAAP and JAAP, lipid biomarker technologies provided estimates of viable cell abundance. Identifying

the amount and nature of the in situ viable microflora in relation to nitroaromatic contamination established a direct link between the rates of contaminant mineralization observed in the radiorespirometry flasks and the indigenous microbial populations. The nucleic acid biomarkers provided the necessary evidence of a genetic capability for natural attenuation at each site. Biomarkers at both sites provided positive evidence that microbial transformation/ mineralization processes play a substantial role in explosives attenuation at these sites. Rates of TNT and RDX mineralization were very low in LAAP soils, and few significant correlations with geochemical parameters and biomarkers were found. At JAAP mineralization rates were considerably higher, possibly because of higher organic matter content in the soils to support the cometabolic degradation. Furthermore, several nucleic acid probes correlated positively with mineralization rate at JAAP as did the following parameters determined by lipid biomarkers: biomass; abundance of gram-negative, sulfate-reducing, and iron-reducing bacteria; and a sulfite reductase. Aerobic degradation of TNT in LAAP soils was indicated by the presence of two catechol oxygenase gene probes. Therefore, the microbial population contained genes for explosives mineralization. At JAAP presence of a gene for a denitrification enzyme suggested the mechanism for microbial reduction of TNT. Other observed genes supported potential for both anaerobic and aerobic metabolism of the TNT ring. Biomarker techniques provided an effective tool for demonstrating microbial destruction potential in field samples. The rate and extent of degradation and transformation were also estimated. The effectiveness of microbially mediated natural attenuation depends upon site characteristics, the composition and abundance of the viable biomass, and the genetic capabilities of the site microflora. Biomarkers were effective in measuring each of these at LAAP and JAAP.

Modeling

Modeling is essential for (a) conceptualization of the contamination at the site so that proximity to receptors can best be determined, (b) evaluation of factors dominating natural attenuation processes at the site, and (c) prediction of long-term contaminant migration and transformation. A numerical model was applied to the LAAP site using results from the other focus areas, i.e., groundwater monitoring, site capacity determinations, and biomarkers, in addition to local weather data and other published information. A comprehensive computer graphical and integral modeling system, the Department of Defense Groundwater Modeling System (GMS) (Brigham Young University 1996), was used. The model contains tools to facilitate site characterization, conceptualization, geostatistical computations, and postprocessing. The model links transport and water quality models to predict the fate and transport of contaminants. Sensitivity of the model simulations and predictions to input parameters was coupled with the desired level of accuracy to determine the level of detail required for field and laboratory measurements. Contaminant mass was also calculated using the measured and predicted explosives concentrations from the model.

The GMS provided efficient numerical tools to integrate and analyze the complex, multidisciplinary field data into simpler graphic forms that were used

to illustrate fate and transport of the contaminant plumes. The measured and simulated flow data indicated slow subsurface flow at the LAAP site due to the low-permeability media and low hydraulic gradients. The TNT and RDX plumes were virtually static. The simulated flow directions were consistent with the direction of explosives plume propagation. The simulated results indicated that explosives at LAAP may be reduced naturally without posing any threat to offsite receptors. The trigger factors for natural attenuation of explosives at the site including low degree of sorption, low values of the hydraulic conductivity, and low rate of mineralization were evaluated and illustrated. Even though the reduction process is very slow, the plume is confined to a limited area and is not moving significantly. The results of contaminant mass calculations indicated that the initial mass of TNT and RDX was reduced from 47,173 and 70,760 to 907 and 726 kg (52 and 78 to 1 and 0.8 tons), respectively, during 20 years of simulation. The sensitivity analysis suggested that the important model input parameters are the adsorption coefficient rates and the biodecay rates. The predicted results should be adjusted and the calibration processes repeated as new data become available. Significant reductions in mass were also predicted at JAAP, but limited migration of the plume was also predicted.

Objectives of This Report

Three research areas, stable isotopes, biomarkers, and modeling, continued in FY 99 and are the subject of this report. Chapter 2, "Use of Carbon and Nitrogen Stable Isotopes," describes the evaluation of the feasibility of using stable isotope ratios for carbon and nitrogen in either soil or groundwater to monitor natural attenuation of explosives. Chapter 3, "Toxicity Testing of LAAP Subsurface Soils," describes results for a single biomarker milestone completed in FY 99. The objective was to determine whether TNT toxicity accounted for the negative correlation between TNT concentration and overall microbial biomass observed in LAAP aquifer soils. Chapter 4, "Integration of Biomarkers Data into the Model 3DFATMIC," addresses a single modeling milestone completed in FY 99. The objective was to numerically simulate and predict the effects of microbial biomass on groundwater hydrology and fate and transport of TNT and RDX at LAAP and JAAP. Chapter 5, "Conclusions," summarizes results presented in this report.

2 Use of Carbon and Nitrogen Stable Isotopes

Background

Theory

Carbon is composed primarily of two natural isotopes: ^{12}C, which accounts for 98.98 percent of all carbon, and ^{13}C, which accounts for approximately 1.11 percent (Craig 1957; Faure 1986; Hoefs 1987). The relative abundance of ^{13}C is expressed as $\delta^{13}C$ values, which represent the ^{13}C to ^{12}C ratio relative to a standard ratio from the PeeDee Belemnite (PDB) Formation, Upper Cretaceous, South Carolina. Nitrogen is also composed of two isotopes, 99.4 percent ^{14}N and 0.6 percent ^{15}N (Watson 1985). The relative abundance of ^{15}N in a sample is determined relative to that of atmospheric nitrogen.

Isotope ratios are reported as a difference, rather than absolute concentrations, because differences are more descriptive of isotope behavior and can be measured more precisely (Friedman and O'Neil 1977; Lajtha and Michener 1994). These difference values, δ or "del," are calculated using Equation 1 and are expressed in parts per thousand, often referred to as "per mil" and generally denoted as ‰. Compounds that are depleted of the heavy isotope relative to the standard have negative δ values; compounds that are enriched in the heavy isotope have positive δ values.

$$\delta = [(R/ R_s) - 1)] \times 1000 \quad (1)$$

where

R = absolute isotope ratio in the sample

R_s = absolute isotope ratio in the standard

Previous uses

Stable isotope analysis has been used to address a broad range of topics in carbon, nitrogen, oxygen, hydrogen, sulfur, and chlorine cycling. The natural differentiation in stable isotope values, such as $\delta^{13}C$ and $\delta^{15}N$ levels, can be used as tracers for following the long-term dynamics of natural systems (Balesdent and Marlotti 1987; Balesdent and Wagner 1988). In the petroleum industry, petroleum compounds have been linked to the source rock formation from which they migrated by relating the $\delta^{13}C$ values of specific petroleum fractions with those in the kerogen (organic) fraction of the source rock (Stahl 1986). In soil science, soil organic matter turnover rates have been estimated by comparing changes in $\delta^{13}C$ values for different soil organic fractions (Boutton, Harrison, and Smith 1980; O'Brien and Stout 1978), and for determining sources of CO_2 evolved from soil (Reardon, Allison, and Fritz 1979). Stable isotope ratios have also been used to estimate long-term changes in plants and soils (Freeman and Hayes 1992).

Stable isotope analysis has also been employed to trace short-term cycling of organic matter through food chains in ecosystems (Peterson, Howarth, and Garritt 1985; Peterson and Fry 1987). The application of stable isotope analysis of food chains has been developed to study microbial roles in elemental cycling (Blair et al. 1985; Coffin 1989; Coffin et al. 1990; Coffin, Cifuentes, and Elderidge 1994). This approach has been applied to delineate carbon sources that support food chains in complex aquatic ecosystems (Coffin 1989; Coffin et al. 1990; Coffin, Cifuentes, and Elderidge 1994; Cifuentes et al. 1996a) and to analyze nitrogen cycling through food chains (Peterson and Fry 1987; Hoch, Fogel, and Kirchman 1992; Hoch et al. 1996). As a result of limits in technology for application of stable isotope analysis, preliminary studies have focused on the major component of elemental cycles in complex pools with numerous sources. Recently, with the development of gas chromatographs/isotope ratio mass spectrometry (GC/IRMS), research has moved to study cycling of specific compounds in ecosystems (O'Malley, Abrajano, and Hellou 1994; Trust et al. in preparation) and of individual compounds as biomarkers to determine microbial roles in biogeochemical cycles (Pelz et al., in preparation). Combining the stable isotope ratio measurement with the high resolution of capillary GC has resulted in a technique that allows measurement of the isotope ratios for individual compounds within a mixture.

Developments in stable isotope analysis are being employed to evaluate remedial efforts in environments that are contaminated with petroleum hydrocarbons. This technology provides the capability to identify sources of contaminants in ecosystems (O'Malley, Abrajano, and Hellou 1994; Kelley, Trust, and Coffin, in preparation). In situ degradation of organic contaminants is studied using ^{13}C analysis of CO_2 evolving from soil (Cifuentes et al. 1996b; Van de Velde et al. 1995). Finally, specific biomarkers have been used to evaluate the bacterial assimilation of contaminants (Pelz et al., in preparation). The combination of these recent developments provides the ability to determine sources and fates of contaminants in an ecosystem.

The feasibility of using a similar technique to estimate TNT attenuation rates in subsurface soils was explored. Since TNT was manufactured from petroleum-based compounds, the carbon ring in TNT should retain the depleted $\delta^{13}C$ values similar to those that are measured in petroleum compounds. A primary fate of TNT in many environments appears to be bonding to or incorporation with the soil organic fraction rather than mineralization (Pennington et al. 1995, 1997, 1998b); therefore, significant evolution of $^{13}CO_2$ derived from TNT is not expected.

Rationale

TNT in a contaminated area undergoes two major processes, reaction with the soil through which it moves and transport with the groundwater. These processes occur simultaneously to influence the fate of TNT in soil and groundwater systems, but separation of reaction and fate processes may be possible in the laboratory. If so, the reaction and transport components could be more mechanistically included in models.

The stable isotopes ^{13}C and ^{15}N were used as tracers to track the processes that occur during TNT degradation. Dual tracers allow measurement of attenuation whether the process is governed by nitro/amino functionality, aromatic ring/toluene functionality, or both. The following two strategies were used: carbon-based changes in the soil and nitrogen-based changes in solution.

Soil carbon. Measuring changes in C isotopic ratios in groundwater is not practical or feasible because of the limited dissolved organic carbon (DOC) typically present. However, incorporation of TNT or covalent bonding of amino-daughter products of TNT to soil humic material may result in a change in the $\delta^{13}C$ of the soil during or after binding. Several factors influence the determination of such measurements:

a. Scant data are available for $\delta^{13}C$ values for TNT from different sources. Furthermore, soil $\delta^{13}C$ values vary due to the origin of parent material and the variety of biological-chemical processes possible.

b. Changes in $\delta^{13}C$ values may occur very slowly under field situations.

c. The amount of TNT-derived carbon binding to soil, and hence the change in $\delta^{13}C$ of the soil carbon after attenuation, may be too small to measure relative to the native carbon levels in the soil. Low-carbon soils would have less "masking" ability than high-carbon soils and would, in theory, require less difference in soil and TNT ^{13}C values to detect changes. Deep aquifer soils, however, may contain very low percentages of organic carbon, resulting in detection difficulties.

d. The amount of TNT-derived carbon that a soil can bind may be "limited" by the humic content, and therefore the carbon levels, in the soil. High-carbon soils would be expected to bind more than low-carbon soils.

Solution nitrogen. Measuring changes in the nitrogen isotopic ratio in soil is not practical because of the many sources of nitrogen present that would distort the test results. However, measuring changes in the nitrogen ratio of the TNT specifically is promising. The amino reduction products of TNT are believed to bond directly to the soil humic material through the amino functional group (Thorn 1997). Therefore, one or two of the nitro groups on the TNT must first be reduced to amino groups. The reduction of nitro groups, whether chemical or biological, may result in isotopic fractionation due to reaction-rate differences between ^{15}N and ^{14}N. If so, a relatively greater percentage of ^{14}N should be reduced and involved in reactions with either humic material or clay. This process would leave relatively more ^{15}N in the TNT remaining in the groundwater system. In effect, the $\delta^{15}N$ values of TNT in groundwater would evolve toward the heavy isotope of nitrogen. Advantages of using the isotopic signature of TNT in groundwater are that groundwater samples are easier to obtain than subsurface soils samples, and groundwater is a well-mixed system relative to the soil. A potential disadvantage is that concentrations of TNT may be too low to obtain reliable $\delta^{15}N$ values. Regardless of the mechanism, changes in the $\delta^{15}N$ values of TNT in groundwater toward heavier nitrogen would indicate that some form of attenuation is occurring.

Two laboratory studies were conducted. The first tracked the degradation of a known amount of TNT in soil over time, measuring the $\delta^{13}C$ and $\delta^{15}N$ values for TNT. The second laboratory study looked at the $\delta^{13}C$ and $\delta^{15}N$ values for TNT in a soil system where an unlimited amount of TNT was available to the system. The objective of both studies was to determine whether change in the $\delta^{13}C$ and $\delta^{15}N$ values for TNT could be determined as the TNT degraded.

Two field studies were conducted: one soil and one groundwater. In the soil study, a number of samples were collected from LAAP and analyzed to determine the TNT concentration and the $\delta^{13}C$ value. The objective was to determine if a change in the $\delta^{13}C$ value for soil carbon could be measured in a real sample. In the second field study, groundwater samples were collected along transects at LAAP with the concentration and $\delta^{15}N$ value for TNT determined. The objective was to look at the change in the $\delta^{15}N$ value for TNT as TNT migrated with the groundwater.

Objective

The objective of this work was to evaluate the feasibility of using ^{13}C and ^{15}N stable isotope analyses as an analytical tool for monitoring natural attenuation at explosives-contaminated sites. Laboratory studies were conducted to determine if changes in the $\delta^{13}C$ and $\delta^{15}N$ values for TNT in solution and the $\delta^{13}C$ values for the soil carbon could be measured as TNT proceeded through its degradation and attenuation process. Studies were conducted using water from LAAP to determine whether changes in the $\delta^{13}C$ and $\delta^{15}N$ values for TNT in groundwater could be measured as a function of time or distance as the TNT plume migrated through the soil. Studies were conducted using soil samples from LAAP to

determine if the $\delta^{13}C$ values for the soil carbon changed over time as a result of exposure to and attenuation of TNT.

Methods and Materials

Instrumental analyses

HPLC analysis. Soil and water extracts for high-performance liquid chromatographic (HPLC) analysis were diluted 1:4 volume per volume (v/v) with water, filtered through a Millex SR 0.5 mm filter (Millipore Corporation, Bedford, MA), and analyzed by reverse phase (RP)-HPLC. The chromatographic system consisted of a Spectra Physics 8800 programmable pump (Spectra Physics, Inc., San Jose, CA) operated in isocratic mode, a Spectra Physics Spectra 100 variable wavelength detector (Spectra Physics, Inc., San Jose, CA) set to 254 nm, and a Dynatech LC-241 autosampler (Dynatech Corporation, Baton Rouge, LA). The analytes were separated on an LC-8 (150 mm × 3.9 mm) column (Waters Corporation, Bedford, MA) eluted with a binary eluant of isopropanol/water (15/85, v/v) at a flow rate of 1.4 mL/min. The column temperature was maintained at 28 °C with a column oven.

GC-IRMS analysis. Stable carbon and nitrogen isotope analysis of nitroaromatics in soil and water extracts were conducted with a Varian Star 3400 CX gas chromatograph (GC) (Varian Instruments, Harbor City, CA) equipped with a Finnigan Magnum ion trap mass spectrometer (ITMS) (Finnigan Corporation, San Jose, CA) and a Finnigan Delta-S isotope ratio mass spectrometer (IRMS) (Finnigan Corporation, San Jose, CA). The analytes were separated on an SPB-1 fused silica capillary column (15 m × 0.32 mm ID, 0.25 mm film thickness) using a temperature program 100 °C to 250 °C at 8.3 °C/min. The effluent from the column was split, 10 percent going to the ITMS for peak identification and the remainder to the IRMS where the analytes were combusted in line at 940 °C to CO_2 and N_2 for isotope analysis. For $\delta^{15}N$ analysis the capillary transfer line was submerged in liquid nitrogen to remove carbon gases from the N_2. The standard for nitrogen was atmospheric N_2 and the standard for carbon was PDB. The detection limits were 1 mg C and 3 mg N and the precision was ±0.2 ‰ for $\delta^{15}N$ and ±0.1 ‰, for $\delta^{13}C$.

Dual inlet IRMS analysis. Soil samples were analyzed isotopically by a modified Dumas combustion method that converts organic carbon and organic nitrogen to CO_2 and N_2 for mass spectral analysis (Macko 1981). Soil samples were placed in quartz tubes with Cu and CuO, evacuated, and sealed. The quartz tubes were then heated to 850 °C at a rate of 4500 °C/hr, kept at 8500 °C for 2 hr, and cooled to room temperature at a rate of 600 °C/hr. The slow cooling cycle ensured that any oxides of nitrogen were decomposed to N_2. The CO_2 was separated from N_2 by cryogenic distillation. The N_2 gas was then analyzed on a Nuclide 3-60-RMS. In turn, CO_2 gas was analyzed on a Finnigan MAT 252 IRMS (Finnigan Corporation, San Jose, CA). The standard for nitrogen was

atmospheric N_2 and the standard for carbon was PDB. The detection limits were 1 mg C and 3 mg N and the precision was ±0.2 ‰ for $\delta^{15}N$ and ±0.1 ‰ for $\delta^{13}C$.

Calibration experiment. A series of standard solutions of Standard Analytical Reference Material (SARM) grade TNT (Army Environmental Center (AEC), Aberdeen Proving Ground, MD) were prepared in acetonitrile (AcN) with concentrations ranging from 2.0 mg/L to 1,000 mg/L. Each standard was analyzed by GC/IRMS and the $\delta^{13}C$ value for TNT was determined. The relationship between concentration and the $\delta^{13}C$ value for TNT was computed and a minimum concentration threshold where reliable data could be obtained was determined.

TNT solutions

An aqueous solution of SARM grade TNT was prepared by placing 250 mg of TNT into 4 L of MilliQ grade water and stirring for 3 days. The solution was filtered to remove any undissolved TNT, then analyzed by HPLC. The resulting concentration was determined to be 49.5 mg/L. This solution was used to spike the samples in Incubation Study 1.

A second aqueous solution of TNT was prepared by placing 3 g of technical grade TNT (Eastman Kodak, Rochester, NY) into 20 L of tap water. The solution was stirred for 10 days, then filtered and analyzed by HPLC. The resulting concentration was determined to be 101 mg/L. This solution was used to spike samples in Incubation Study 2.

Incubation study 1: Isotope ratio of TNT-amended soil over time

Two soils were chosen for the incubation study, one from Charlton, NH, and the other from LAAP. The Charlton soil was a sandy loam with 1.3 percent total organic carbon. The LAAP soil was primarily sand with a total organic carbon content of 0.012 percent. Both soils were oven dried at 105 °C, then sieved through a No. 20 sieve (0.84 mm). Samples were prepared by placing 25 g of soil into a 50-mL test tube (50 samples of each soil were prepared). A 2.0-mL aliquot of tap water was added to each sample in order to rewet the soil. The samples were incubated in the dark at room temperature for 3 days to allow the restart of microbiological activity. A 4.0-mL aliquot of aqueous TNT (49.5 mg/L) solution was added to 35 of the 50 samples. A 4.0-mL aliquot of MilliQ grade water was added to the remaining 15 samples to be used as blanks. The samples were returned to the dark and incubation was allowed to continue at room temperature (22° ± 2°C).

Sampling events took place immediately after preparation (time zero), then at 1, 3, 7, 14, 21, and 28 days. For each sampling event, three replicates of each spiked soil were chosen at random. Blanks were sampled in triplicate on days 0, 3, 14, and 28. Each soil sample was quantitatively transferred from the incubation tube to an extraction thimble and extracted by Soxhlet for 24 hr with

AcN at a rate of six cycles per hour. The final extract was diluted volumetrically to 250 mL. The soil was allowed to air-dry overnight to allow the AcN to evaporate, then oven-dried at 105 °C overnight to remove any remaining water or solvent. Extracts were analyzed for concentration of TNT and its transformation products by HPLC-UV as described previously and by GC/IRMS for $\delta^{13}C$ and $\delta^{15}N$ values for TNT. The dried soil was analyzed by dual inlet IRMS for $\delta^{13}C$ and $\delta^{15}N$ values of the soil.

Incubation study 2: Unlimited contaminant source, maximum loading of soil

Due to limited quantities of LAAP and Charlton soils for this study, a soil collected at Hanover, NH, was oven dried (105°C) and sieved as described previously. The Hanover soil was a silty loam containing 1.5 percent total organic carbon. Each of 48 slurry samples was prepared by placing 25 g of soil into 250-mL glass jars and adding 6.0 mL of tap water to rewet the soil. The samples were then allowed to incubate in the dark at room temperature for 3 days. A 100-mL aliquot of aqueous TNT solution (101 mg/L) was added to 39 of the 48 samples. To the remaining nine samples, 100 ml of tap water was added. The samples and blanks were shaken on a wrist action shaker for 20 minutes, then returned to the dark for incubation. Also prepared at this time were three control samples containing only 100 mL of the TNT spiking solution plus 6 mL of tap water (no soil).

The spiked soils were sampled at days 0, 7, 14, 21, 28, and 56. Blanks were sampled at days 0, 21, and 56. At each sampling event, all of the jars were shaken for 20 minutes on a wrist action shaker, then centrifuged for 45 minutes at 2,000 rpm (RCF = 682 × g). Three samples were randomly selected and placed to one side for analysis. On the days that blanks were analyzed, three blanks were also selected. For the remaining samples, the aqueous phase was decanted, weighed, then discarded. A fresh 100-mL aliquot of the technical grade TNT solution was then added to each sample. The samples were again shaken for 20 minutes, then returned to the dark.

The samples selected for analysis were centrifuged for 45 minutes at 2,000 rpm. The aqueous phase was poured off, weighed, then transferred to a brown glass jar. The aqueous phase was analyzed by HPLC as described previously to determine the concentration of TNT and the 2-amino- and 4-amino-dinitrotoluene reduction products. Three subsamples of each soil phase were placed in aluminum tins and weighed. The subsamples were air-dried for several days until a constant weight was achieved. The dried subsamples were extracted with AcN and the extracts analyzed by HPLC as described previously to determine the concentration of the analytes of interest and by GC/IRMS for the $\delta^{13}C$ and $\delta^{15}N$ values for TNT. The remainder of the soil was extracted by Soxhlet extractors for 24 hr with AcN, dried, then analyzed by dual inlet IRMS for the $\delta^{13}C$ and $\delta^{15}N$ values.

Isotope ratios across the contaminated plume at LAAP

Four transects were drawn across the LAAP site between wells in the area of high concentration within the TNT plume and wells along the leading edge of the plume (Figure 1). Table 1 describes the specifics of each transect. Six 1-L groundwater samples were collected from each of the six wells selected in April 1998 and then again in September 1998.

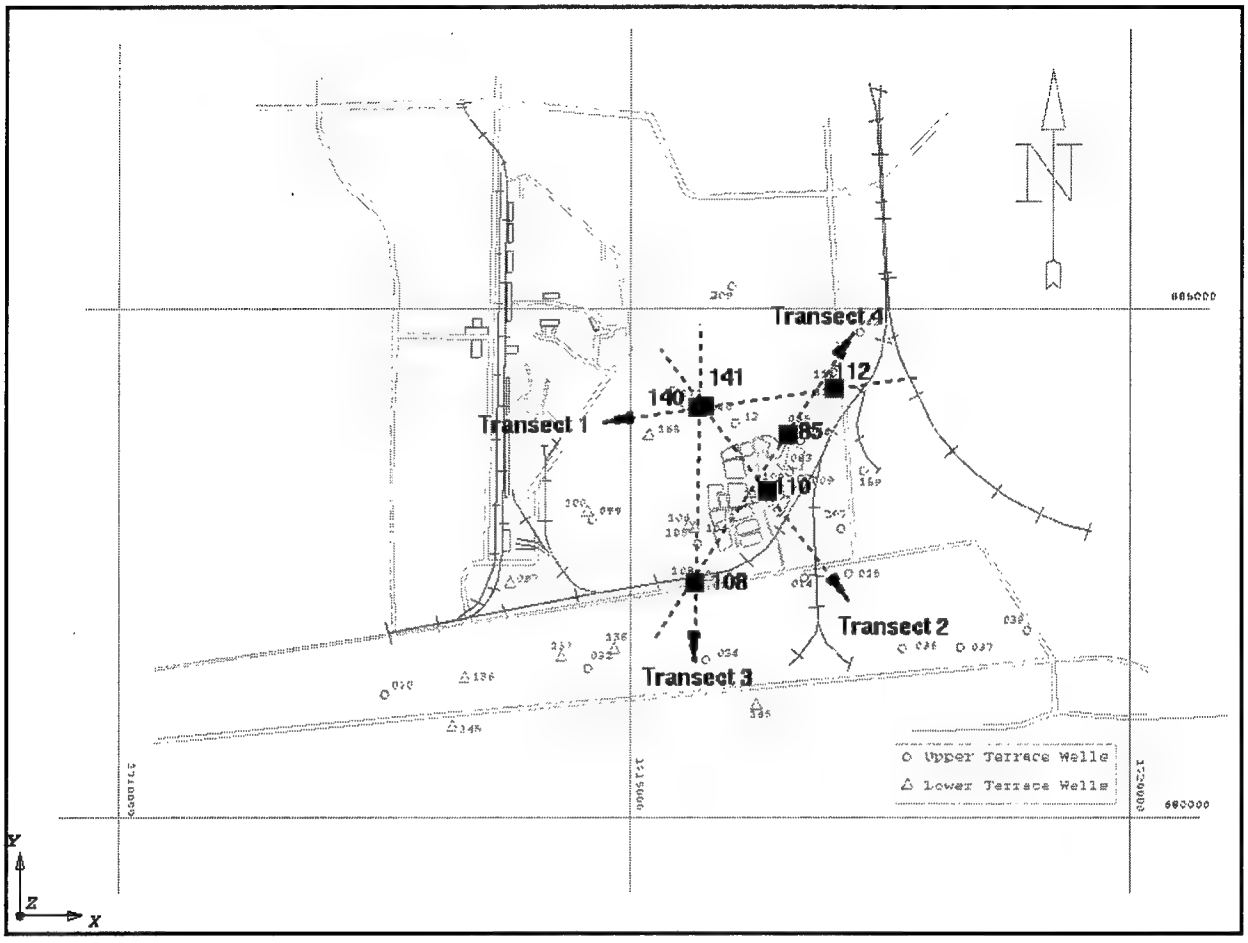

Figure 1. Transect between monitoring wells sampled for determination of $\delta^{15}N$ values for TNT in groundwater at LAAP

Table 1
Description of Transects Along Which Groundwater Samples Were Collected

Transect Number	Well in the Plume	Well at Leading Edge	Aquifer
1	141	112	Lower
2	141	110	Lower
3	140	108	Upper
4	085	108	Upper

The TNT was extracted from the groundwater using Bond Elut EVN 200 mg/3 cc solid-phase extraction cartridges (Varian, Harbor City, CA). Duplicate 1-L samples were extracted for the plume samples and duplicate 2-L samples were extracted for the leading edge samples. The groundwater samples were passed through the cartridges at a rate of approximately 10 mL/min. Because of the high TNT concentration of the samples, two cartridges were placed in series to prevent losses of analyte resulting from any breakthrough. The TNT was recovered from the cartridges with 5.0 mL of AcN and the concentrations determined by HPLC. The extracts were then analyzed by GC/IRMS to determine the $\delta^{13}C$ and $\delta^{15}N$ values for TNT.

Isotope ratios in soils at LAAP

Soil core samples were collected from six locations at LAAP using a cone penetrometer with a split-spoon sampler (see Appendix A for list of sample locations). At each location, subsamples were obtained from 5 to 14 different depths ranging from approximately 1.5 to 20 m for determination of concentration of TNT and stable isotope ratios for extractable TNT and soil carbon.

The soil samples were air-dried, ground with a mortar and pestle, then passed through a No. 20 sieve (0.84 mm). A 2.0-g subsample of each soil was extracted with AcN. The extract was analyzed by HPLC to determine the concentration of TNT and the mono-amino-dinitrotoluenes. An additional subsample of each soil was washed twice with 10 mL of AcN for 18 hr with ultrasonication, air-dried, then analyzed by dual inlet IRMS to determine the $\delta^{13}C$ and $\delta^{15}N$ values for the soil.

Results and Discussion

Method development

The initial task for this project was to develop sample preparation techniques that would allow isolation and preconcentration of TNT, but would not introduce isotopic fractionation, which would confound results. Two methods, one for soil samples and one for water samples, were developed for exhaustive extraction without isotopic fractionation.

For soil samples, the Soxhlet extractor was chosen as the most effective means of extraction. Previous studies demonstrated that the efficiency of the Soxhlet extractor is equal to that of ultrasonication (Jenkins and Leggett 1985; Jenkins and Grant 1987). Although Soxhlet extractions are slow, this technique has the unique characteristic that the analytes are completely separated from the soil in the extractor. The solvent reservoir/collection vessel and sample holder are physically separated. In the case of ultrasonic extraction recommended in the standard EPA method, the sample and extraction solvent are in the same vessel. Therefore, complete (100 percent) removal of the solvent in ultrasonic extraction

is not possible. Some residual solvent remains with the soil sample, and this solvent will contain some of the analytes. With Soxhlet extraction, once passed to the collection vessel, the analytes cannot again come into contact with the soil. The results of an extraction kinetics experiment showed that a 24-hr Soxhlet extraction was required to obtain 99.9 percent recovery of TNT from field-contaminated soil using AcN as the extraction solvent.

For extraction of TNT from water samples, solid phase extraction (SPE) was the method of choice. The groundwater was passed through SPE cartridges at 10 mL/min and the analytes were recovered with 5 mL of AcN. The recovery for TNT was 99.8 percent.

Spiked soil and water samples were prepared and processed using the methods described previously. The extracts from these samples were analyzed to determine the $\delta^{13}C$ and $\delta^{15}N$ values for TNT. These results were compared to the $\delta^{13}C$ and $\delta^{15}N$ values for TNT dissolved in solution at the same concentration as the extracts (control solution). Results for both techniques showed that no isotopic fractionation was occurring during the sample preparation processes (Table 2)

Table 2
Comparison of $\delta^{13}C$ and $\delta^{15}N$ Values for TNT Subjected to Different Sample Preparation Processes

Sample Preparation	$\delta^{13}C$	$\delta^{15}N$
Control solution	-28.2	3.41
SPE cartridge extract from groundwater	-28.0	3.42
Soxhlet extract from soil	-28.2	3.41

The concentration of TNT in moist soil samples is known to decrease with time under certain environmental conditions (Grant, Jenkins, and Golden 1993). Thus the mass of TNT recovered over a time course experiment will decline with time. The objective of this calibration experiment was to determine if the $\delta^{13}C$ value for TNT in solution varied as a function of concentration or mass injected into the analyzer. Above 4.0 mg/L (or 4.0 ng injected), the $\delta^{13}C$ value for TNT was unaffected by the changing concentration (Table 3). The result for the 1.96-mg/L (2.0-ng) standard shows a dramatic increase in the $\delta^{13}C$ value for TNT. These data indicate that below a concentration threshold of 4 mg/L, the $\delta^{13}C$ value for TNT is concentration dependent. Therefore, all analyses required an injection mass of 4 ng or greater.

Table 3
Concentration of TNT in Solution Versus $\delta^{13}C$ Value for TNT

Concentration of TNT, mg/L	Mass of TNT injected, ng	$\delta^{13}C$ value for TNT, ‰
1,001	1,000	-31.9
501	501	-31.2
250	250	-28.5
125	125	-29.6
62.5	62.5	-27.9
31.3	31.3	-26.2
15.6	15.6	-25.4
7.8	7.8	-29.4
3.9	3.9	-34.5
1.96	2.0	-17.2

Incubation study 1: Isotope ratio of TNT-amended soil over time

For the Charlton soil the concentration of TNT decreased rapidly over the first 7 days (Figure 2). The concentration of the 2-amino- and 4-amino-dinitrotoluene (2ADNT and 4ADNT) reduction products increased during the first 7 days. After day 14, the concentrations of all of the analytes decreased with time. These concentration changes have been observed by others (Grant, Jenkins, and Golden 1993; Pennington et al. 1995). The mass balance calculations indicated that recoverable TNT and transformation products decreased by 70 percent from the original mass of TNT in just 21 days (Figure 3). A much smaller decrease in concentration of TNT and a small increase in the concentration of the amino-dinitrotoluenes was observed in the LAAP soil. The total loss of extractable TNT and transformation products was 37 percent over 77 days (Figure 3). No significant change in the $\delta^{13}C$ value for the extractable TNT (Figures 4 and 5) or for the $\delta^{13}C$ of the soil carbon was observed (Figure 6).

Although the concentration of extractable TNT decreased during incubation (Figures 2 and 3), no changes could be detected in the $\delta^{13}C$ value for TNT (Figures 4 and 5). A measurable change in the $\delta^{13}C$ value for the soil was sought as the TNT became irreversibly bound to the soil organic matter. No change in the $\delta^{13}C$ value for the soil could be measured over the length of the experiment (Figure 6). Thorne reports that after transformation and immobilization of most of the TNT in finished compost, the highest percentage of TNT remaining in association with the organic matter was 0.2 percent weight per weight (w/w) (Pennington et al. 1997, 1998b). The total percent carbon in the soils used in these studies ranged from 0.012 percent for LAAP soil to 1.3 percent for the

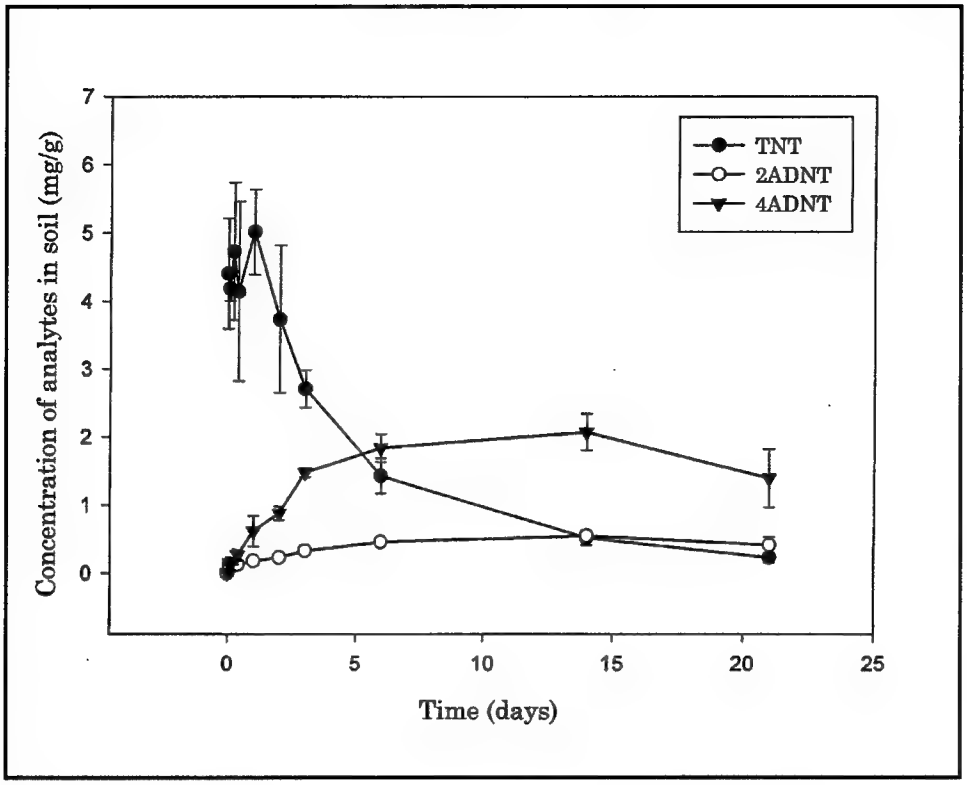

Figure 2. Concentration of TNT, 2ADNT, and 4ADNT over time for the experiment using Charlton soil

Charlton, NH, soil. If TNT became bound to the soil organic matter at a level of 0.2 percent (w/w), the mass of carbon resulting from the TNT would be only 19 mg in a 2-g soil sample. This represents only 0.074 percent of the total carbon present in the sample, which is a difference too small to measure.

Incubation study 2: Unlimited contaminant source, maximum loading of soil

At time zero TNT was the only analyte in the system and was present solely in the aqueous phase. By day 7, the analyte composition consisted of both TNT and the amino-dinitrotoluenes, primarily TNT, which was distributed between the aqueous and soil phases. The total TNT mass had decreased to 90 percent of the original (Table 4). By day 21 the percent of amino-dinitrotoluenes had increased, but the primary component of the system was still the TNT in the solution phase. The total mass had increased to 105 percent of the original mass and remained unchanged through day 56 (Table 4). The mass changes and redistribution of the analytes translated to concentrations of extractable TNT, 2ADNT, and 4ADNT of 25 mg/g, 0.1 mg/g, and 0.4 mg/g, respectively, after 14 days. At day 14, the concentration of the analytes in the aqueous phase was 91 mg/L, 0.2 mg/L, and 0.7 mg/L for TNT, 2ADNT and 4ADNT, respectively.

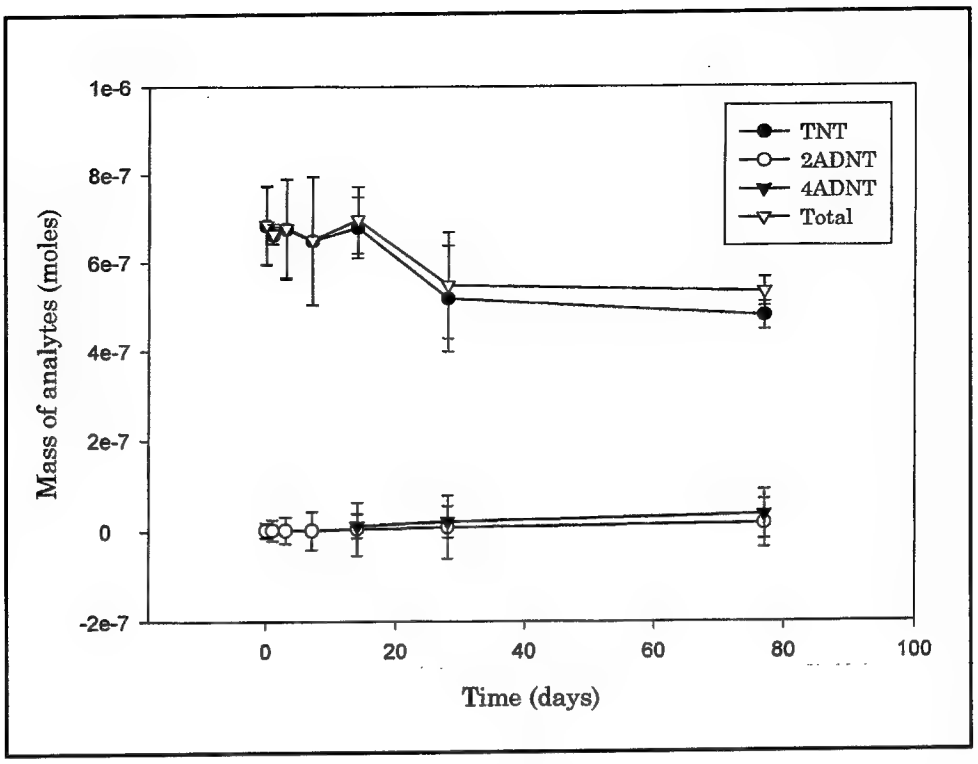

Figure 3. Mass of TNT, 2ADNT, 4ADNT and total mass recovered over time for the experiment using LAAP soils

All of these concentrations remained relatively unchanged through 56 days. The data plotted in Figure 7 showed definite trends in the change in concentration of TNT over time. The isotope data suggest a possible trend; however, the precision of the measurements indicates great uncertainty.

Isotope ratios across the contaminated plume at LAAP

The concentration of TNT in the groundwater near the source region of the plume ranged from 1,870 mg/L to 5,110 mg/L (Table 5). Concentrations along the leading edge ranged from 116 mg/L to 772 mg/L. A significant difference between the concentration of TNT in the center of the plume and that at the leading edge was evident for both the upper and lower terraces. No significant differences were found in the $\delta^{13}C$ values for TNT. As predicted, the $\delta^{15}N$ values for TNT increased as the TNT moved away from the source area in the upper aquifer, but for the lower the opposite was observed. Transport may not be simply away from the center of the plume. The cause for the observed trend is unclear. A second sampling of the wells at LAAP was performed in September 1998. Again the TNT was extracted from the groundwater and the concentration and $\delta^{15}N$ values determined (Table 6). These data showed the same concentration gradients along each transect. The statistical analysis of the data

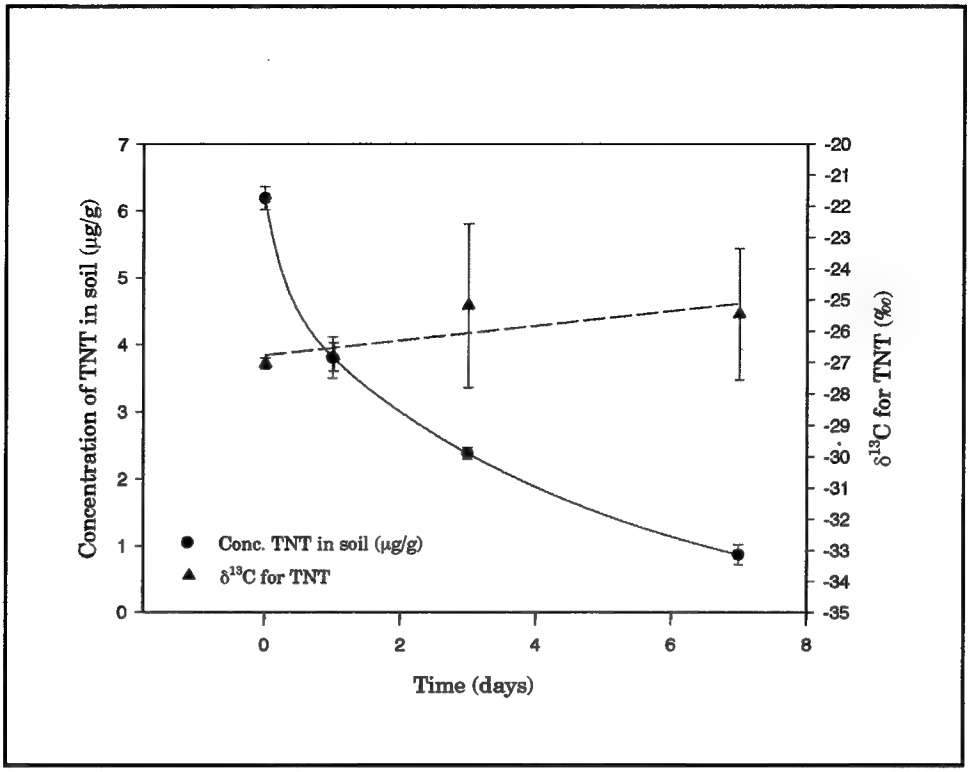

Figure 4. Concentration of TNT in the soil and the $\delta^{13}C$ value for extractable TNT over time for the experiment using Charlton soil

(Table 7) using the Student's t-test showed a clear increasing trend in the $\delta^{15}N$ values for TNT, indicating that the TNT was being fractionated and was becoming isotopically heavier. These results suggest that as the TNT migrated through the soil, a process removed the lighter isotopic fraction of TNT from the groundwater. Using $\delta^{15}N$ measurements of TNT from groundwater to monitor the fate or natural attenuation of TNT in the environment is promising. The current limitation on their use is the lack of kinetics data for the rate of change of the $\delta^{15}N$ value for TNT as a function of rate of change of the TNT concentration as it degrades naturally.

Isotope ratios in soils at LAAP

TNT was detected in most subsamples at concentrations from 0.1 mg/g to 1.8 mg/g. Analysis indicated no difference between the $\delta^{13}C$ values for blank soil samples and those contaminated with TNT. The extractable TNT was also analyzed for $\delta^{15}N$; however, due to the low concentration of TNT in the extracts, reliable measurements could not be obtained.

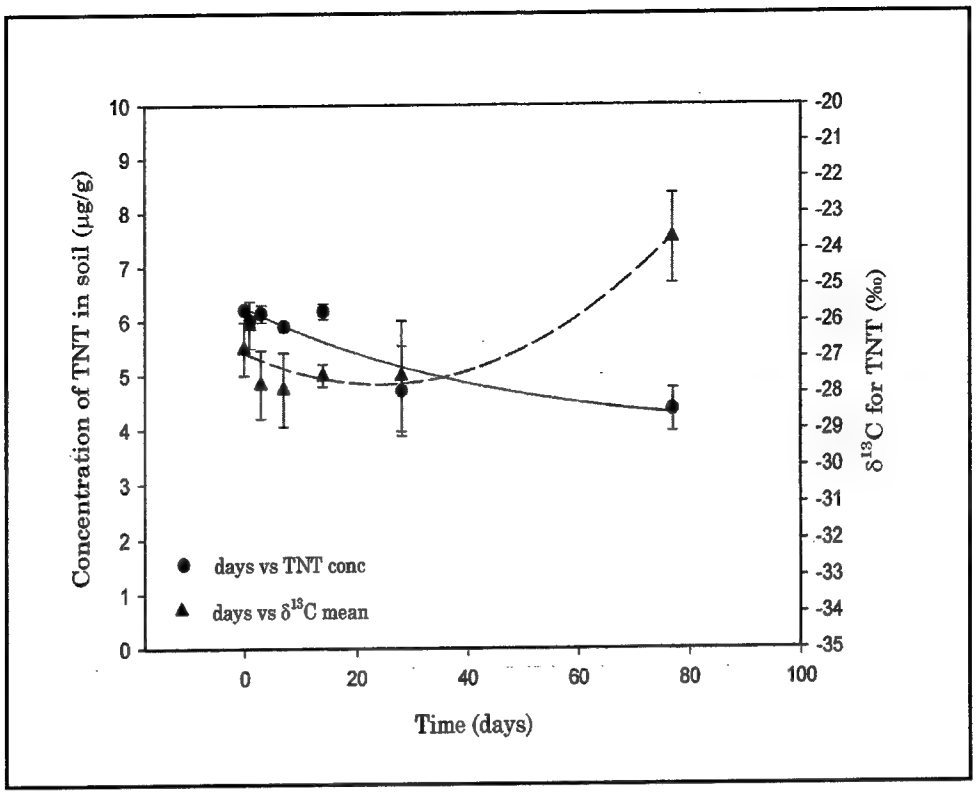

Figure 5. Concentration of TNT extracted from the soil and the $\delta^{13}C$ value for extractable TNT over time for the experiment using LAAP soil

Summary

The $\delta^{15}N$ data suggested the feasibility of monitoring natural attenuation of TNT using $\delta^{15}N$ measurements. In two field experiments, increasing $\delta^{15}N$ values for TNT in the groundwater were observed as the TNT migrated through the soil. Data across three of the four transects at LAAP showed a significant increase in the $\delta^{15}N$ values for TNT with decreases in concentrations. These data are consistent with the reduction of the nitro groups to amino functional groups, followed by binding of the amino or di-amino products to the soil organic carbon (Pennington et al. 1998b). Both steps in the process involve reactions of the nitrogen groups; therefore, measuring $\delta^{15}N$ values is a very promising monitoring tool for natural attenuation of TNT and its transformation products in groundwater.

In contrast to the positive results from the $\delta^{15}N$ data, the $\delta^{13}C$ data proved to be less useful. The TNT quantities at which reliable isotope ratio values can be obtained were 250 ng and 750 ng for the $\delta^{13}C$ and $\delta^{15}N$ analyses, respectively. Below 4 ng TNT injected on the column, the $\delta^{13}C$ measurement was mass dependent. This threshold value translated to a soil concentration of 20 mg TNT/g when extracting a 2-g sample with 10 mL of solvent. This is two orders of magnitude higher than the 0.1-ug/g detection limits of the EPA standard

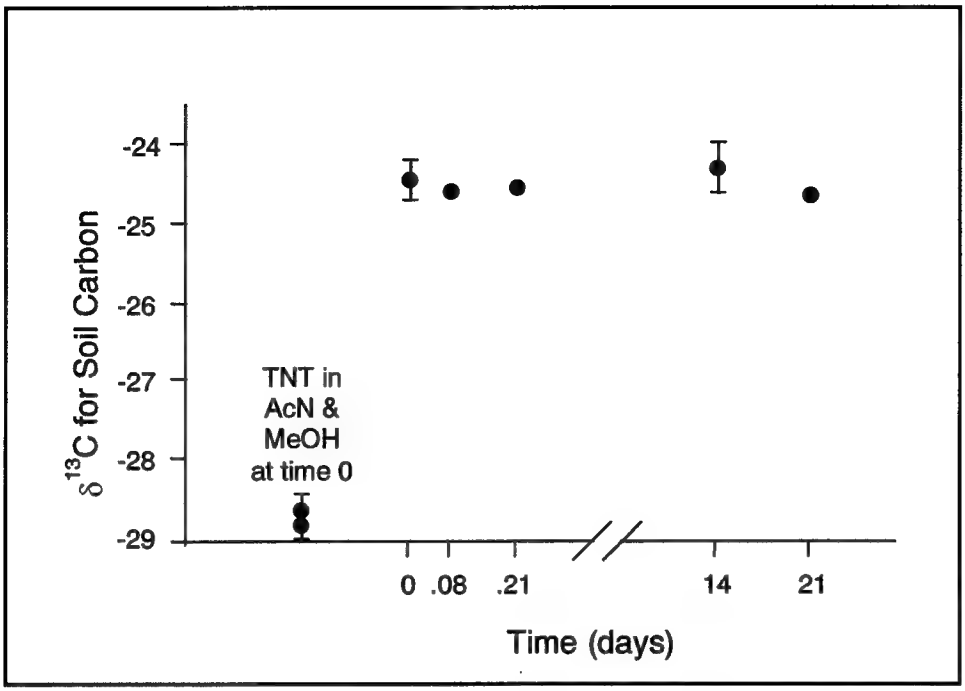

Figure 6. Concentration of TNT extracted from the soil and the $\delta^{13}C$ value for extractable TNT over time for the experiment using LAAP soil

Table 4
Mass Recovered from Aqueous and Soil Phases over Time in Incubation Study 2, moles ± Standard Deviation

Analyte and Medium	Mass Recovered, moles					
	0 Day	7 Days	14 Days	21 Days	28 Days	56 Days
TNT in aqueous phase	4.36E-05 ±5.27E-07	3.81E-05 ±5.19E-07	4.39E-05 ±1.57E-07	4.63E-05 ±3.93E-07	4.59E-05 ±1.66E-07	4.54E-05 ±4.69E-07
2ADNT in aqueous phase	0.00	5.13E-08 ±1.05E-08	1.15E-07 ±1.79-08	7.73E-08 ±7.73E-09	6.54E-08 ±4.77E-09	1.20E-07 ±1.91E-08
4ADNT in aqueous phase	0.00	1.91E-07 +3.18E-08	4.03E-07 +4.89E-08	2.29E-07 +3.24E-08	1.56E-07 +1.51E-08	2.66E-07 +4.48E-08
TNT in soil	0.00	7.74E-08 ±4.93E-05	3.72E-07 ±1.52E-04	3.87E-07 ±6.50E-05	3.51E-07 ±6.38E-05	3.97E-07 ±1.91E-04
2ADNT in soil	0.00	3.40E-09 ±7.96E-07	1.99E-09 ±8.43E-07	4.31E-09 ±8.09E-07	1.45E-09 ±3.51E-07	1.77E-09 ±7.93E-07
4ADNT in soil	0.00	2.11E-08 ±4.89E-06	1.43E-08 ±5.48E-06	9.84E-09 ±1.96E-06	6.76E-09 ±1.40E-06	7.93E-09 ±2.69E-06
Total recovered	4.36E-05	3.85E-05	4.49E-05	4.75E-05	4.65E-05	4.62E-05
Total added	4.47E-05	4.33E-05	4.55E-05	4.53E-05	4.44E-05	4.36E-05
Total moles recovered, %	97.6	88.8	98.6	104	105	106

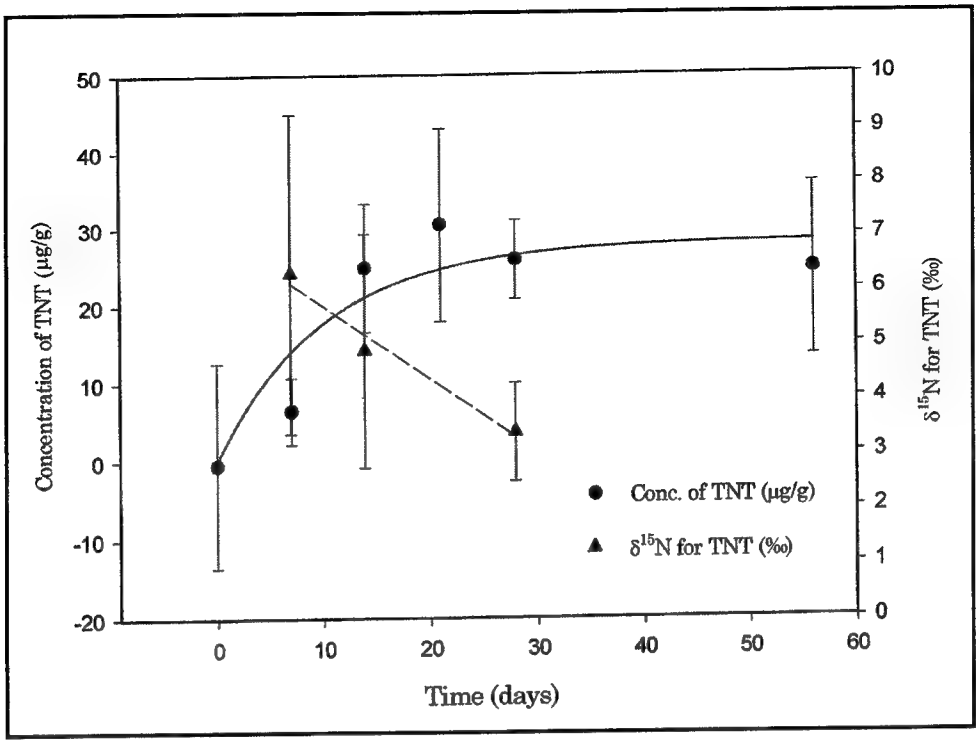

Figure 7. Concentration and $\delta^{15}N$ of extractable TNT over time for Incubation Study 2

Table 5
Concentration and Stable Isotope Data for TNT Extracted from LAAP Groundwater Collected in April 1998

Location of Sample	Well	Transect	Terrace	TNT Concentration mg/L	$\delta^{13}C$, ‰	$\delta^{15}N$, ‰
Plume	MW 141	1	Lower	2,660	-20.3	13.2
		2	Lower	2,660	-20.3	13.2
	MW 140	3	Upper	1,870	-20.9	9.44
	MW 085	4	Upper	5,110	--[1]	11.3
Leading Edge	MW 112	1	Lower	116	-21.9	6.14
	MW 110	2	Lower	772	-18.5	9.30
	MW 108	3	Upper	542	-17.8	14.74
		4	Upper	542	-17.8	14.7

[1] Data for this point were not available due to a chromatographic interference.

Table 6
Concentration and $\delta^{15}N$ Values for TNT Extracted from LAAP Groundwater Collected in September 1998

Location of Sample	Well	Transect	Terrace	TNT Concentration mg/L	$\delta^{15}N$, ‰
Plume	MW 141	1	Lower	2,870	13.3
		2	Lower	2,870	13.3
	MW 140	3	Upper	1,920	9.7
	MW 085	4	Upper	5,950	10.1
Leading Edge	MW 112	1	Lower	75.1	17.3
	MW 110	2	Lower	1,060	10.0
	MW 108	3	Upper	567	13.8
		4	Upper	567	13.8

Table 7
Statistical Comparison of $\delta^{15}N$ Values for TNT Extracted from LAAP Groundwater Collected in September 1998

Sample	Mean	SE	F	Pooled SE	t
Transect #1					
141	13.26	0.956	6.32	1.83	-3.16
112	17.35	2.402			
Transect #2					
141	13.26	0.956	1.42	1.05	4.42
110	9.98	1.137			
Transect #3					
140	9.69	0.592	3.10	0.48	-12.22
108	13.85	0.336			
Transect #4					
085	10.10	0.689	4.20	0.54	-9.77
108	13.85	0.336			
Critical t Values					
t Values (df = 3)	**90%**	**95%**	**99%**	**99.9%**	
	2.13	2.78	4.60	8.60	
Critical F Values					
F Values (df = 3.3.)	**90%**	**95%**			
	29.00	9.28			

method for explosive (EPA 1998). The concentration of TNT in the soil samples collected at LAAP ranged from 0.1 mg/g to 1.8 mg/g. Even where the soil was in contact with an unlimited source of TNT for several weeks, the concentration of TNT adsorbed to the soil reached only 25 mg/g, which was just within the detection threshold. Therefore, a change in the $\delta^{13}C$ value for the soil carbon resulting from the addition of TNT to the soil carbon cannot be measured due to the detection limitations of the analysis.

Sample preconcentration would improve detection, but may introduce greater measurement uncertainty. Preconcentration may address the limitation for analytes in solution, but not in soil. One of the key objectives was to measure changes in the $\delta^{13}C$ value for the soil carbon as TNT becomes irreversibly bound to the soil organic matter. As discussed, the change in mass of carbon in a soil sample resulting from TNT binding with organic matter in the soil is less than 0.1 percent. This is far too small a change to measure precisely.

The prediction that no changes in the carbon isotope ratio would be detected for TNT in solution, even with large changes in concentration of TNT, was verified. Changes in $\delta^{13}C$ in soil carbon could not be detected as TNT became irreversibly bound to the soil organic matter. In incubation study 1, the concentration of TNT decreased by sixfold over a very short time, but no significant change in the $\delta^{13}C$ value for the soil was measured. In incubation study 2, the concentration of extractable TNT increased to 25 mg/g over 77 days, but no significant changes were measured in the $\delta^{13}C$ for soil carbon. Attenuation processes occur, but do not fractionate the carbon isotopes. Soil samples collected at LAAP showed no significant differences between samples from different locations or for samples for different depths at the same locations.

Conclusions

The objective of this work was to evaluate whether $\delta^{13}C$ and/or $\delta^{15}N$ stable isotopes measurements of TNT could be used as a monitoring technique for natural attenuation of TNT. The data clearly did not support the use of $\delta^{13}C$ measurements because changes in the $\delta^{13}C$ value for soil organic matter were too small to detect. However, changes in the $\delta^{15}N$ values for TNT in groundwater could be detected across a contaminant plume. In order to utilize such a measurement, a quantitative assessment of the rate and magnitude of the change in the $\delta^{15}N$ value of TNT as a function of the following will be required: TNT degradation rate, the concentration gradient of TNT across a plume, the contact time with the soil, and the mass of soil through which the TNT has passed.

3 Toxicity Testing of LAAP Subsurface Soils

Introduction

Analysis of the microbial communities in LAAP soils resulted in a significant negative correlation between TNT concentration and overall viable biomass (Pennington et al. 1999b). This study was conducted to test the hypothesis that this correlation was due to TNT toxic effects.

The dual soil toxicity test developed by Azur Environmental (Azur Environmental, Carlsbad, CA) was used to examine the potential acute and mutagenic toxicities of contaminants in soil. The Azur Environmental assays are based on responses of the marine luminescent bacterium *Vibrio fischeri,* which produces visible light as a by-product of its normal metabolic processes. When these normal processes are altered by exposure to a toxicant, light output is changed. Therefore, differences in light production are directly proportional to the "bioreactivity" of the sample. In the MicroTox assay, bioreactivity is expressed as the effective concentration that causes a 50 percent reduction in light output by the microorganism (EC_{50}%). The MutaTox assay employs a dark variant of *Vibrio fischeri.* When the dark strain contacts a DNA-damaging compound (genotoxic agent), light is produced in direct proportion to the amount of genotoxic agent present. Phenol is used as a positive control to ensure that the dark variant is responsive to a toxic compound. To determine whether or not a contaminant can be transformed to a more reactive (genotoxic) compound by enzymatic activation, mouse liver hepatic enzymes (S-9 solution) is used, and benzo[a]pyrene is employed as a positive control to ensure that the S-9 mixture is working properly.

Materials and Methods

Solid phase MicroTox assay

Soil samples from LAAP Site 6-2 (Pennington et al. 1999b), which had been archived by freezing at -80 °C, and a control sediment sample from Brown's

Lake, Vicksburg, MS, were tested. Dry weight equivalent of 7 g of each soil was mixed and placed into a 50-mL beaker to which was added 35 mL of solid-phase diluent (3.5 percent NaCl solution in distilled water). The mixture was stirred for 10 min with a Teflon-coated magnetic stirring bar. The MicroTox test was then run on each soil following the instructions provided by the manufacturer. Following incubation of the samples, the solids were separated using a filter column supplied with the test. Light output from the filtrate for each sample was read using an Azur Environmental Model 500 Analyzer. The output file for each sample was corrected for sample dry weight based on moisture determinations made for triplicate soil samples. The Brown's Lake soil served as the uncontaminated control.

MutaTox test

Phenol and benzo[a]pyrene reagents were used as positive controls for the MutaTox test. The phenol solution was prepared by adding 0.2 g of reagent grade phenol to 20 mL of a reconstitution solution provided with the test. The benzo[a]pyrene solution was prepared by adding 0.01 g of benzo[a]pyrene to 20 mL of dimethylsulfoxide (DMSO). Both reagents were stored at 4 °C until used.

To prepare the soil extracts, 1 g of each soil was extracted with 2 mL of DMSO, vortexed, sonicated for 2 min in an ultrasonic bath, and then incubated for 3 hr at room temperature. The soils and extractants were separated by centrifuging for 5 min at 250 RCF), and the extractants were decanted and tested according to the manufacturer's instructions for the MutaTox test alone and in combination with the S-9 solution, both employing the dark strain of *Vibrio fischeri*. The light output was measured at 16, 20, and 24 hr of incubation on the Azur Environmental Model 500 Analyzer. Results were corrected for the dry weight value of the soil sample. The Brown's Lake soil served as the uncontaminated control.

Data analysis

Data were analyzed using the Spearman Rank Order Correlation and Pearson Product-Moment Correlation in the SigmaStat Statistical Software Package (McClave and Dietrich 1982; Federle et al. 1986; Jandel 1995). To establish a direct relationship between the toxic effect and the measurement system used, the MicroTox toxicity data were expressed as toxicity units (TU) using the formula $TU = r(100)/EC_{50}$, where r is the correlation coefficient (Ghirardini et al. 1998).

Results and Discussion

No substantial expression of mutatoxicity was found in any of the samples. When compared to the uncontaminated Brown's Lake sediment, all but two of the LAAP Site 6-2 samples were more toxic (Table 8). However, when compared with the findings of Ghirardini et al. (1998) for Venice lagoon and inner canal sediments and with those of Simini et al. (1995) for JAAP soils, LAAP Site 6-2 toxicity values were low. The only exception was the LAAP Site 6-2 soil from 15.2 m, which had a toxicity in the low to moderate range in comparison with the materials from Venice and JAAP. Toxicity (as TU) typically correlates positively with contaminant concentration; i.e., toxicity increases in proportion to increases in contaminant concentrations. However, when toxicity results were compared with contaminant concentration and microbial biomass, no correlation was found (Table 9).

Table 8
MicroTox™ Results from Seven LAAP Subsurface Soils and Uncontaminated Brown's Lake Sediment

Depth, m	EC_{50}%	EC_{50}% Range	Slope	Correlation Coefficient, *r*	TU
LAAP Soils					
3.0	0.55	0.42-0.73	3.90	0.97	176.4
6.1	2.56	2.37-2.78	4.17	0.99	38.7
8.2	1.16	0.90-1.40	2.51	0.95	81.9
10.4	1.14	0.85-1.53	1.44	0.88	77.2
12.2	0.43	0.33-0.58	0.88	0.93	216.3
15.2	0.27	0.19-0.37	0.81	0.91	337.0
17.4	1.22	0.68-2.19	0.56	0.78	64.0
Brown's Lake Sediment					
0	1.46	1.31-1.54	1.48	0.99	68.0

Note: EC_{50}% values, slopes, and correlation coefficients were used to compute toxicity units (TU).

Conclusions

Most of the LAAP subsurface soils from Site 6-2 showed a greater toxicity than the "clean" (no detectable explosives or other organic contaminants) lake sediment; however, except for the LAAP Site 6-2 soil from 15.2 m, the level of toxicity was generally lower than that previously reported at an explosive- and heavy metal-contaminated site (JAAP). No correlation was found between toxicity values and contaminant chemistry or microbial biomass. Therefore, the

correlation previously observed between biomass and TNT concentration was not due to toxicity.

Table 9
Summary of Linear Correlations (Spearman r and Pearson Product) Between Toxicity Units (TU) and Explosives Contamination and Microbial Biomass in LAAP Subsurface Soil

Variable	Number of samples	Spearman		Pearson	
		r	p-value	Coefficient	p-value
Explosives Contaminant, ng g^{-1}					
RDX	7	0.36	0.432	0.11	0.809
TNT	7	0.09	0.848	-0.12	0.793
TNB	7	0.11	0.819	-0.13	0.782
HMX	7	-0.15	0.751	-0.25	0.580
2ADNT	7	-0.02	0.967	-0.25	0.590
Microbial Biomass					
Viable, pmol PLFA g^{-1}	7	0.57	0.180	0.28	0.549
Total, ng DNA g^{-1}	7	0.05	0.907	0.30	0.509

Note: Correlation coefficient was considered significant at the 95% level of confidence if $p < 0.005$.

4 Integration of Biomarkers Data into the Model 3DFATMIC

Introduction

Accounting for flow, transport, and reaction processes that control bacterial growth and migration patterns would enhance modeling capabilities for monitored natural attenuation of explosives. 3DFATMIC (3-Dimensional Subsurface Flow, Fate and Transport of Microbes and Chemicals Model) is a numerical model that has been developed to simulate the coupled reactive contaminant transport and microbial growth. The code was developed as part of a cooperative agreement between the EPA National Management Research Laboratory in Ada, OK, and Pennsylvania State University (Chen 1995; EPA 1987).

Baveye and Valocchi (1989) describe three different conceptual models that may be used in mathematical models for growth and transport of microbes in soil and groundwater. These include a biofilm model, a microcolony model, and a macroscopic model. Because the geometric parameters describing the size of biofilms or microcolonies are not widely found in the literature, the microbial-chemical reactions in 3DFATMIC are formulated with a macroscopic approach (Chen 1995). The macroscopic approach neglects the microscopic configuration (the way in which the microorganisms are grouped together on the solid surface) and assumes that the microorganisms are attached to and grow on the surface of solid particles (see for example, Borden and Bedient 1986).

The biomarker study provided biomass data, which may be used as input parameters in 3DFATMIC (Table 10 for LAAP and Table 11 for JAAP). The data were collected as snapshot samples (collected at the same time) at a few horizontal locations and at one time at both sites.

The model 3DFATMIC was selected to provide time-series distribution of biomass over domains. The predicted results from modeling could provide information on the spatial and temporal distribution of biomass and, with help

Table 10
Biomass Data for Selected Location at Area P, LAAP

Sample	Depth, m	Biomass, pmol PLFA/g[1]	Cells/g (Estimated)[2]
1-1a	7.3152	11.36	2.8E+05
1-1b	9.144	3.16	7.9E+04
1-1c	10.3632	7.86	2.0E+05
1-4a	7.80288	61.21	1.5E+06
1-4b	13.1064	2.27	5.7E+04
2-1a	7.80288	4.49	1.1E+05
2-1b	10.2108	5.90	1.5E+05
2-1c	18.8976	1.71	4.3E+04
2-3a	5.54736	2.28	5.7E+04
2-3b	13.35024	0.36	9.1E+03
2-3c	17.43456	1.30	3.2E+04
2-5a	7.49808	6.70	1.7E+05
2-5b	12.16152	1.11	2.8E+04
2-5c	13.99032	1.88	4.7E+04
3-2a	6.18744	7.92	2.0E+05
3-4a	11.49096	6.67	1.7E+05
3-4b	18.25752	37.13	9.3E+05
3-5a	7.3152	28.56	7.1E+05
3-5b	12.71016	7.14	1.8E+05
3-5c	19.2024	16.71	4.2E+05
3-7a	11.09472	38.67	9.7E+05
3-7b	14.81328	4.04	1.0E+05
3-7c	17.03832	66.26	1.7E+06
4-5a	9.35736	3.22	8.1E+04
4-5b	20.45208	13.63	3.4E+05
6-2a	1.524	163.6	4.1E+06
6-2b	3.048	16.8	4.2E+05
6-2c	4.572	17.4	4.3E+05
6-2d	6.096	0.6	1.4E+04
6-2e	7.62	0.9	2.3E+04
6-2f	8.2296	2.4	6.1E+04
6-2g	9.144	1.8	4.4E+04
6-2h	10.3632	0.6	1.6E+04
6-2i	12.25296	11.9	3.0E+05
6-2j	13.04544	1.3	3.2E+04
6-2k	13.716	2.9	7.2E+04
6-2l	15.24	3.4	8.6E+04
6-2m	17.49552	9.8	2.5E+05
6-2n	18.288	5.0	1.2E+05

[1] Biomass determined by measuring phospholipid fatty acid (PLFA) in soil samples.
[2] Based on the assumption that 1 pmole of PLFA is equivalent to 2.5×10^4 cells of a typical subsurface bacterium (Balkwill et al. 1988).

Table 11
Sample Location of Biomass for L1 Site, JAAP

Sample	Depth, m	Biomass, pmol PLFA/g[1]
S3T1VP1	0.23	13395
S3T1VP2	0.99	730
S3T1VP3	1.95	138
S3T1VP4	2.82	21
S3T1VP5	4.33	56
S3T1VP6	14.9	54
S4T1VP2	0.23	15245
S4T1VP3	1.14	1167
S4T1VP4	1.78	258
S4T1VP5	2.39	ns
S4T1VP6	3.14	78
S4T1VP7	5.58	80
S5T1VP2	0.23	ns
S5T1VP3	0.87	47
S5T1VP4	1.54	51
S5T1VP5	2.66	62
S5T1VP6	3.38	29

[1] ns = Not significant.

from other biomarker data, conclusions about effects of biomass on hydrogeology and fate and transport of explosives. In short, the objective of this task was to numerically simulate and predict the effects of microbial biomass on groundwater hydrology and fate and transport of TNT and RDX at LAAP and JAAP.

Code Description

3DFATMIC can be used to investigate saturated-unsaturated flow, contaminant transport, combined flow and transport, and the fate and transport of microbes and chemicals in the groundwater environment. The special advantages of 3DFATMIC are flexibility and versatility in modeling a wide range of problems. This model can be used for the following conditions and simulations: (a) heterogeneous and anisotropic media consisting of as many geologic formations as desired; (b) both spatially distributed and point sources/sinks that are spatially and temporally dependent; (c) the prescribed initial conditions by input

or by simulating a steady state version of the system under consideration; (d) the prescribed transient concentration over Dirichlet nodes; (e) time-dependent fluxes over Neumann nodes; (f) time-dependent total fluxes over Cauchy nodes; (g) variable boundary conditions of evaporation, infiltration, or seepage on the soil-air interface for the flow module and variable boundary conditions of inflow and outflow for the transport module automatically; (h) two options of treating the mass matrix, consistent and lumping; (i) three options (exact relaxation, under- and over-relaxation) for estimating the nonlinear matrix; (j) automatic time-step size reset when boundary conditions or sources/ sinks change abruptly; (k) Galerkin weighting or upstream weighting for the advection term in the transport module; (l) mass balance checking over the entire region for every time-step; and (m) possibility of modifying the code for different field situations.

3DFATMIC requires extensive data input parameters. To facilitate data inputting, Pennsylvania State University has developed a program[1] to link 3DFATMIC to the DoD GMS for the U.S. Army Engineer Waterways Experiment Station. The linkage program provides a user friendly pre- and postprocessing program for inputting numerous data parameters into 3DFATMIC.

For the transport module, concentrations of the following seven components are included: substrate, oxygen, nitrate, nutrient, microbe 1 with aerobic respiration, microbe 2 with anaerobic respiration, and microbe 3 with both aerobic and anaerobic respiration. When oxygen and nitrate are present, the chemical reactions are processed by reducing oxygen or nitrate. Therefore, oxygen and nitrate act as electron acceptors. Processes including advection, dispersion, diffusion, chemical transformation, and biodegradation are modeled.

For transport simulations, the bulk density, the longitudinal and transverse dispersivities, the tortuosity, the chemical transformations rate, and the distribution coefficient in each material must be provided. The kinetic parameters regarding microbial-chemical reactions are needed for the calculation of biodegradation. Among these parameters, the growth rate changes significantly with temperature and may be specified as a function of temperature.

Application

Two significant problems were encountered in applying 3DFATMIC to the biomass data from LAAP and JAAP. First, 3DFATMIC requires more extensive data than were available from the two sites (Tables 10 and 11; Figures 8 and 9).

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[1] Gour-Tsyh (George) Yeh, Jing-Ru Cheng, M. Zakikhani, and J. C. Pennington. (1988). "POP3DMIC: Users manual of pre- or post-processor for 3DFATMIC (3DFATMIC: user's manual of a three-dimensional subsurface flow, fate and transport of microbes and chemical version 1.0) using GMS version 2.1 (Groundwater Modeling Systems)," U. S. Army Engineer Waterways Experiment Station, Vicksburg, MS (unpublished).

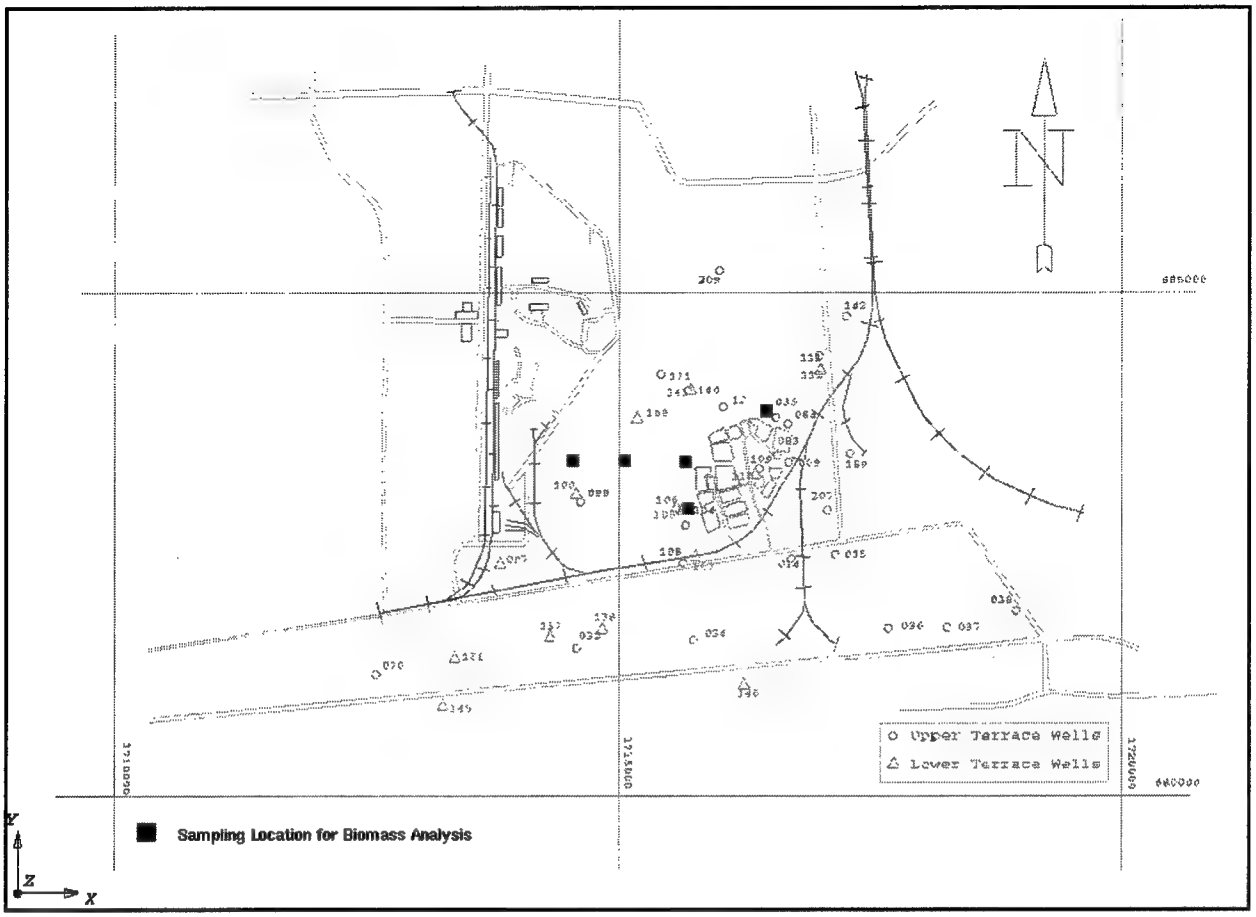

Figure 8. Sampling locations of biomass data at LAAP

The biomass data were collected as snapshot samples at a few horizontal locations at both sites (Figures 8 and 9). The spatial and temporal distributions of available biomass data were not enough for model calculations and predictions. The discrete data collected at a site usually are interpolated/extrapolated to estimate properties at intermediate points at each numerical grid. The number of available data points (biomass data) was inadequate for generating data at other locations in the relatively large domains. The second problem was with the numerical coding of 3DFATMIC. The model is very new and was not tested previously against actual field data. The application of 3DFATMIC to LAAP data indicated that the simulation (model run) does not converge for the field input parameters. Investigation to resolve this coding error of 3DFATMIC is in progress at Pennsylvania State University.

Conclusions

In spite of numerous options available in the 3DFATMIC model, the major deficiency with its present form is the inability to simulate interaction between

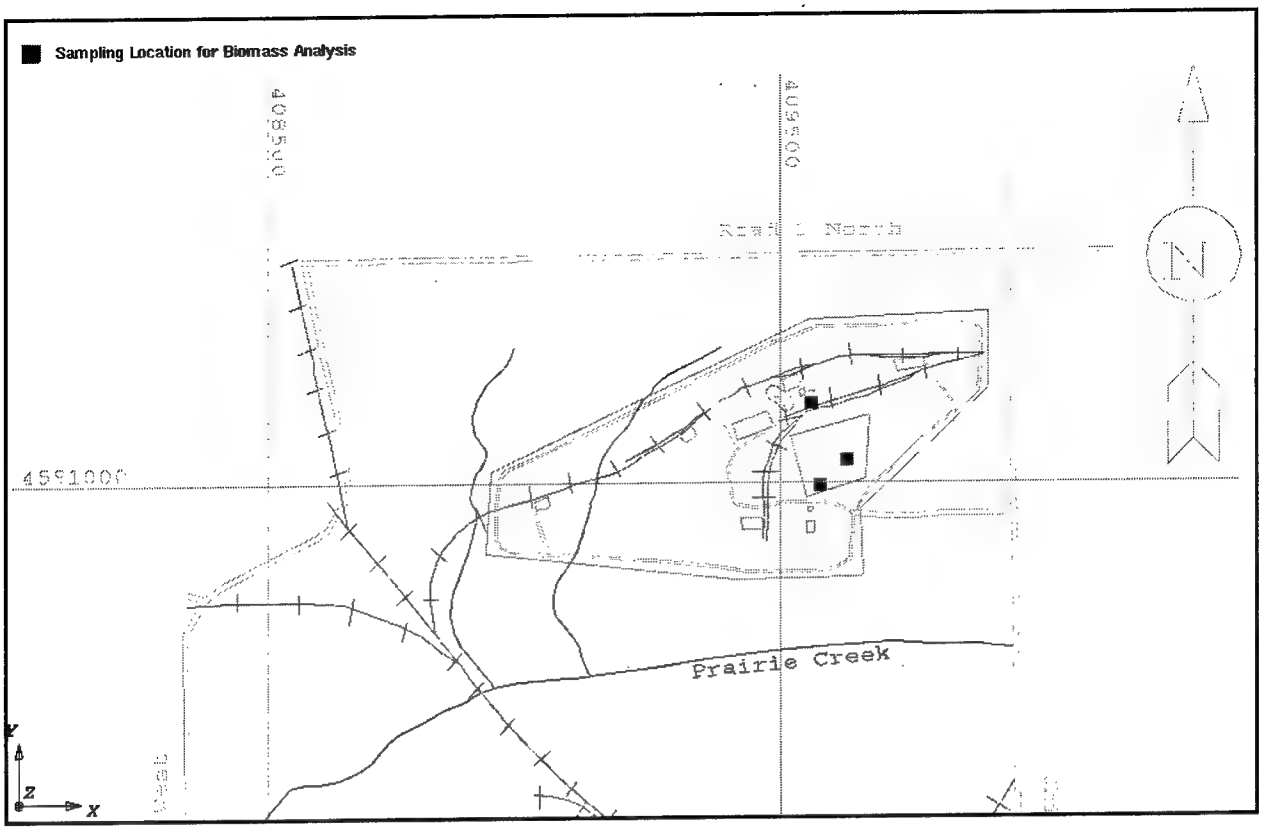

Figure 9. Sampling locations of biomass data at JAAP

explosives and their by-products. However, for biomass simulations, a corrected version of the current 3DFATMIC may be sufficient. Predictive information on spatial and temporal distribution of biomass relative to explosives at LAAP contamination may be generated by 3DFATMIC once problems with the model are resolved and additional biomass data become available.

5 Conclusions

Changes in stable isotope ratios for carbon in soil organic matter were too small to detect and relate to changes in TNT concentration. Therefore, this approach is not feasible for monitoring natural attenuation of TNT. However, changes in stable isotope ratios for nitrogen in TNT found in groundwater were detected across the contaminant plume at LAAP. Monitoring natural attenuation with nitrogen stable isotope ratios may be feasible once the rate and magnitude of isotope changes are related quantitatively to TNT attenuation rates.

The significant negative correlation between TNT concentration and viable biomass in LAAP aquifer soils could not be related to toxicity of TNT to the indigenous microflora. Toxicity assays were restricted to vertical profile samples from only one location. At this site, toxicity was generally lower than those observed at JAAP.

To enhance the usefulness of biomarker data, a new groundwater model, 3DFATMIC, was applied to microbial biomass data from LAAP. The model was designed to simulate spatial and temporal distribution of biomass in the context of hydrogeology and fate and transport of the contaminant. Due to the limited size of the available data set and a problem with the numerical code, 3DFATMIC was unsuccessful with the LAAP biomass data at this stage of the model development. Predictive simulations may be generated by the model once the problems with the model are resolved and additional biomass data become available. Perhaps this will be possible as LAAP continues to monitor the site.

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Appendix A
List of Soil Samples from Louisiana Army Ammunition Plant Used in Stable Isotope Tests

Sample number designations refer to natural attenuation project (NA), transect number, location along transect, and depth in meters. For example, NA2-1U-7.8 refers to the second transect, first sampling location on that transect, and a depth of 7.8 m. U indicates upper strata, L indicates lower strata, VP indicates a vertical profile series of samples.

NA2-1U-7.8
NA2-1U-10.2
NA2-1L-14.4
NA2-1L-18.9
NA2-3U-5.5
NA2-3L-13.4
NA2-3L-17.4

NA4-2VP-1.6
NA4-2VP-3.1
NA4-2VP-4.6
NA4-2VP-5.4
NA4-2VP-7.7
NA4-2VP-9.1
NA4-2VP-10.7
NA4-2VP-12.2
NA4-2VP-13.7
NA4-2VP-15.1

NA4-5VP-1.5
NA4-5VP-3.1
NA4-5VP-4.6
NA4-5VP-6.1
NA4-5VP-7.6
NA4-5VP-9.4
NA4-5VP-10.7
NA4-5VP-14.0
NA4-5VP-15.2
NA4-5VP-16.8
NA4-5VP-18.3
NA4-5VP-20.5

NA6-2VP-1.5
NA6-2VP-3.0
NA6-2VP-4.6
NA6-2VP-6.1
NA6-2VP-7.6
NA6-2VP-8.2
NA6-2VP-9.1
NA6-2VP-10.4
NA6-2VP-12.3
NA6-2VP-13.0
NA6-2VP-13.7
NA6-2VP-15.2
NA6-2VP-17.5
NA6-2VP-18.3
NA6-3VP-6.3
NA6-2VP-10.5
NA6-2VP-13.0
NA6-2VP-15.9
NA6-2VP-17.9

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This report, which is a follow-on to the report "Natural Attenuation of Explosives in Groundwater at DoD Sites: Interim Report," presents results of a study to evaluate the feasibility of using stable isotopes of carbon and nitrogen in soil or groundwater to monitor natural attenuation of explosives. The report also includes continuation results from two research areas, toxicity testing of surface soils at the demonstration site and integration of biomarker data into the model 3DFATMIC (3-Dimensional Subsurface Flow, Fate, and Transport of Microbes and Chemicals). Results of stable isotope studies indicated that changes in carbon isotope ratios were too small to detect. Therefore, this approach is not feasible. However, changes in nitrogen ratios in TNT found in groundwater were detected across the contaminant plume. Once the rate and magnitude of these changes are related quantitatively to TNT attenuation rates, this approach may be feasible for monitoring attenuation processes. In toxicity tests, the significant negative correlation between TNT concentration and viable biomass in aquifer soils at the Louisiana Army Ammunition Plant, Doyline, LA, could not be related to toxicity of TNT to the indigenous microflora. In the modeling research area, the 3DFATMIC model exhibited a problem in the numerical code, and the amount of biomass data proved too small. Predictive simulations may be possible with the model once the code is revised and additional biomass data become available.

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Groundwater modeling	TNT	

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